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The *Drosophila* wing hearts originate from pericardial cells and are essential for wing maturation

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ABSTRACT

In addition to the heart proper, insects possess wing hearts in the thorax to ensure regular hemolymph flow through the narrow wings. In *Drosophila*, the wing hearts consist of two bilateral muscular pumps of unknown origin. Here, we present the first developmental study on these organs and report that the wing hearts originate from eight embryonic progenitor cells arising in two pairs in parasegments 4 and 5. These progenitors represent a so far undescribed subset of the Even-skipped positive pericardial cells (EPC) and are characterized by the early loss of *tinman* expression in contrast to the continuously Tinman positive classical EPCs. Ectopic expression of Tinman in the wing heart progenitors omits organ formation, indicating a crucial role for Tinman during progenitor specification. The subsequent postembryonic development is a highly dynamic process, which includes proliferation and two relocation events. Adults lacking wing hearts display a severe wing phenotype and are unable to fly. The phenotype is caused by omitted clearance of the epidermal cells from the wings during maturation, which inhibits the formation of a flexible wing blade. This indicates that wing hearts are required for proper wing morphogenesis and functionality.

Introduction

Unlike in vertebrates, where an elaborate closed blood vessel system extends throughout the whole body, insects possess only one vessel, the tubular heart, in their otherwise open circulatory system. Once the hemolymph has left the heart, it moves freely between the internal organs and can not be directed into narrow body appendages such as antennae, legs or wings. To ensure sufficient hemolymph supply of these appendages additional circulatory organs evolved (Pass, 2000; Pass et al., 2006). In *Drosophila*, circulation in the wings is maintained by the so-called wing hearts (Krenn and Pass, 1995), a pair of autonomous muscular pumps located bilaterally in the scutellum, the dorsal elevation of the second thoracic segment. Due to this location, they are also referred to as scutellar pulsatile organs (Miller, 1950). Although known for many years, no developmental studies on the origin or morphogenesis of these organs have been performed. Probably, this was due to the lack of available methods to track their differentiation. However, studies on the origin of the thoracic somatic muscles in *Drosophila* (Lawrence, 1982) and comparative anatomical investigations in insects (Krenn and Pass, 1995) suggested that the wing hearts originate from the cardiac mesoderm or from the heart itself.

In a previous study, we identified an enhancer region of the *Drosophila hand* gene that is able to drive reporter gene activity in the wing hearts (Sellin et al., 2006). In the present work, we have used this reporter to identify the embryonic anlagen of the wing hearts and to elucidate the dynamics of their postembryonic development with *in vivo* time lapse imaging. We found that the anlagen of the *Drosophila* wing hearts indeed derive from the cardiac mesoderm but, astonishingly, not from the muscular cardioblast lineage. Instead, they represent a so far undescribed subpopulation of the well-known Even-skipped (Eve) positive pericardial cells (EPCs) (Carmena et al., 2002, 1998a,b; Han and Bodmer, 2003; Han et al., 2002; Park et al., 1998; Su et al., 1999).

In addition to their unknown origin, little is known about the contribution of wing hearts to wing morphogenesis and functionality. After eclosion, wings are unfolded by a sudden influx of hemolymph and subsequently undergo maturation (Johnson and Milner, 1987). During this process, the epidermal cells that until then bonded the dorsal and ventral wing surfaces enter programmed cell death, delaminate from the cuticle, and disappear into the thorax (Kimura et al., 2004). Subsequently, the cuticles of the intervein regions become tightly bonded to form a flexible wing blade, while the cuticles of the vein regions form tubes, lined by living cells (Johnson and Milner, 1987), through which hemolymph circulates in mature adult insects. Measurements of hemolymph flow in adult butterflies showed that wing hearts function as suction pumps that draw hemolymph out of the wings starting shortly after wing unfolding

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(Wasserthal, 1976). We therefore tested whether wing hearts might play a role in wing maturation by generating flies lacking wing hearts. Our findings demonstrate that the delaminated epidermal cells are removed from the wings by the hemolymph flow generated by the wing hearts. Loss of wing heart function leads to remains of epidermal cells resting between the unbonded dorsal and ventral wing surfaces which results in malformation of the wing blade and flightlessness. We conclude that wing hearts are essential for wing maturation and, thus, for acquiring flight ability in *Drosophila*.

Materials and methods

Fly stocks

Oregon R was used as wild type. *hand*-C-GFP, *hand*-C-DsRed and *hand*-C-Gal4 flies were generated using the full-length third intron of the *hand* gene (Sellin et al., 2006). *hand*-$C^{\Delta 395-552}$–GFP and *hand*-$C^{\Delta 395-633}$-GFP are transgenes carrying the full-length third intron with a deletion of the regions 395–552 or 395–633. These reporter lines display GFP expression only in the *tinman* expressing cardioblasts and in the *even-skipped* expressing pericardial cells (data not shown). The vectors used were pH-Stinger (eGFP), pRed H Pelican (DsRed) (Barolo et al., 2000, 2004) and pPTGal4 (Sharma et al., 2002). All transgenic *Drosophila* lines were established by the use of the w^{1118}-line. Gene misexpression was achieved with the UAS-Gal4 system (Brand and Perrimon, 1993) using the following lines: *eme*-Gal4 (from Rolf Bodmer) *twi*-Gal4 (from Alan Michelson), UAS-TIN (from Manfred Frasch) and *hand*-C-Gal4 (our laboratory). UAS-mCD8::GFP (membrane GFP; mGFP), UAS-eGFP, and *30A*-Gal4 were obtained from the Bloomington stock center (BL5137, BL6874, BL1795). The following mutant stocks were used: *eve* meso minus (J49) (Fujioka et al., 2005), $hand^{KO}$ (Han et al., 2006), $zfh1^{2}$ (Lai et al., 1993), $insc^{\Delta 13}$ (Hassan et al., 1998), $numb^{1}$ (Bloomington stock center, BL4096). The *pavarotti* allele $pav^{301-051=E186}$, originally generated by the Klämbt-laboratory (Hummel et al., 1999a,b), was identified in a screen for mutants affecting heart development (Albrecht et al., 2006).

Immunohistochemistry and histological procedures

Immunostainings for β-Galactosidase were performed using the Vectastain Elite ABC Kit (VectorLabs). Primary antibodies were mouse anti-β-Galactosidase (Promega-Z378A) at 1:2000, rabbit anti-β3-Tubulin at 1:1500 (Leiss et al., 1988), rabbit anti-Eve at 1:3000 (Frasch et al., 1987), rabbit anti-GFP (Abcam-ab6556, Cambridge, UK) and mouse anti-GFP (Invitrogen) at 1:600 to 1:2000, mouse anti-Pericardin (Mab3) at 1:5 (from Hybridoma Bank, University of Iowa) (Yarnitzky and Volk, 1995), rabbit anti-Mef2 at 1:1500 (Bour et al., 1995), rabbit anti-Tin at 1:500 (Bodmer, 1993), and mouse anti-Zfh1 at 1:1500 (Lai et al., 1991). All secondary antibodies (Dianova) were used at 1:200. For labeling of nuclei Draq5 (Alexis Biochemicals, Germany) was used. Tissues were incubated for 30 min in dye solution (1:500 in phosphate buffered saline, PBS) and afterwards washed several times. For histological cross sections, specimens were fixed in a mixture of 4% formaldehyde and 1% glutaraldehyde in PBS for 2 h, dehydrated in ethanol and embedded in Epon 812 resin. Semi-thin sectioning (1 μm) was performed on a Leica Ultracut UCT ultramicrotome using glass knives. Sections were staining with toluidine blue at 70 °C for 2 min.

Laser ablation

Stage 16/17 embryos carrying a *hand*-C-GFP construct were prepared as described in Supatto et al. (2005). Ablation was performed using a 40x/1.4 Plan-Apochromat objective on a Zeiss 510 META confocal laser scanning microscope (LSM) equipped with a chameleon titan-sapphire laser (Coherent, USA), adjusted to 6% transmission efficiency at 780 nm. The nuclei of individual cells were irradiated for about 4.5 s using the crop function of the Zeiss AIM software. Operated embryos were kept in PBS until the larvae hatched, then they were transferred to fly vials and incubated at 22 °C for examination as pupae or adults. For ablation in pupae, the puparium was removed with fine forceps only in the region of the scutellum. Pulsed laser light (408 nm, 3 ns duration) emitted from a nitrogen dye laser (Rapp OptoElectronic, Germany) was used to irradiate the wing heart through the cuticle at 3–4 positions, with 30 Hz bursts of 60 s duration focused with a 20x/0.5 Plan-Neofluar objective on a Zeiss Axioskop 2 microscope.

Preparations and image analysis

All dissections were carried out in PBS using fine forceps or a razor blade. For detailed laser scans pupae at 40 h APF or 80 h APF (with the puparium removed) were placed on a cavity microscope slide covered with a drop of Voltalef 10 S oil (VWR, Germany) and sealed with a cover slip. Images were captured with a Zeiss 510 Meta or a Zeiss 5 Pascal LSM. Epifluorescence was observed using either a Leica MZ16FA stereomicroscope or a Zeiss Axioskop 2 microscope, both equipped with UV-illumination and a digital camera. Image capturing was performed with the software package AnalySIS (SIS software, Münster, Germany) or Axiovision (Zeiss, Germany).

Movies and time-lapse studies

For bright field live recordings of the beating wing heart, adults were decapitated and placed on a cavity microscope slide. The region of the wing heart was then covered with a drop of Voltalef 10 Soil (VWR, Germany) and sealed with a cover slip. Movies were recorded with a digital consumer camera using a 10x/0.3 Plan-Neofluar objective on a Zeiss Axioskop 2 microscope. Epifluorescence live recordings of beating wing hearts and clearance of epidermal cells from the wings were performed on a Leica MZ16FA stereomicroscope equipped with UV-illumination and a digital camera. Images were acquired at a rate of 1 in 2 s using the software package AnalySIS (SIS software, Münster, Germany). For faster acquisition of images during wing clearance and better separation of red and green fluorescence, a Zeiss 5 Pascal laser scanning microscope was used at maximum scan speed (~2.5 images per second) in combination with a 10x/0.3 Plan-Neofluar objective. For time-lapse studies of the developing wing hearts, prepupae were adjusted in a slightly upright position on a cavity microscope slide; pupae after removal of the puparium on a regular microscope slide. To prevent dehydration a chamber was built of moistened stripes of filter paper and a cover slip. Image stacks were recorded at 20-min intervals for about 50 h using a 10x/0.3 Plan-Apochromat objective on either a Zeiss 510 META or a Zeiss 5 Pascal laser scanning confocal microscope. Movies were created from maximum projections of each obtained image stack.

Flight assay

Adults were tested individually between 3 and 24 h after wing unfolding only if their wings were intact. We used a Perspex cube of 30 cm edge length with a circular opening of 15 cm in diameter in the top, which allowed manual operation in the cube. Flies were gently dumped in from a vial through a funnel placed in the center of the opening. Flight ability was scored positive when the flies either landed on the wall or flew in the cube. Flies falling straight to the bottom failed the test. To confirm flightlessness, such animals were mechanically stimulated with a fine brush.

Results

Morphology of wing hearts observed by hand-C-GFP reporter expression

In a previous study, a *hand*-C-GFP reporter was generated (Sellin et al., 2006) that reflects the described *hand* expression pattern (Han and Olson, 2005; Kölsch and Paululat, 2002; Moore et al., 2000) and was found to be active in wing hearts. To confirm that the *hand*-C-GFP reporter is expressed in all cells of mature wing hearts, we examined their morphology based on the signal from the reporter in conjunction with histological sections. In the adult fly, wing hearts are located at the lateral angles of the scutellum, which are joined to the posterior wing veins by cuticular tubes (Figs. 1A–D, O) (Krenn and Pass, 1995; Miller, 1950). Each organ is curved in anterior–posterior direction as well as dorso-ventrally. It consists of about 7–8 horizontally arranged rows of prominent muscle cells, which are attached at their proximal side to a thin layer of cells that has a greater dorsal extension than the muscle cells (Figs. 1C, D). Both cell types are labeled by the reporter. The fine acellular strands that hold the wing hearts to the adjacent epidermal cells (Krenn and Pass, 1995) were not observed to be marked by the reporter (Figs. 1C, D). To demonstrate the location and the beating of wing hearts, movies are provided (Supplementary data, Movies 1 and 2).

Wing hearts develop from embryonic anlagen

We tested the *hand*-C-GFP reporter for expression in earlier stages of wing heart development and found that it is active throughout the entire organogenesis. This enabled us to identify the embryonic anlagen of the wing hearts, which consist of eight progenitor cells located dorsally and anterior to the heart, in two pairs in the second and third thoracic segment from stage 16/17 onward (Fig. 1E). The progenitors exhibit a flattened triangular shape and are interconnected by thin cytoplasmic extensions (Fig. 2J). In addition, the second and the fourth pair of the progenitors are closely associated with the dorsal tracheal branches at their interconnection in the second and third thoracic segments (Figs. 1M, N). The characteristic pairwise arrangement and the connection to the tracheae are retained during the subsequent three larval stages. Proliferation starts at about the

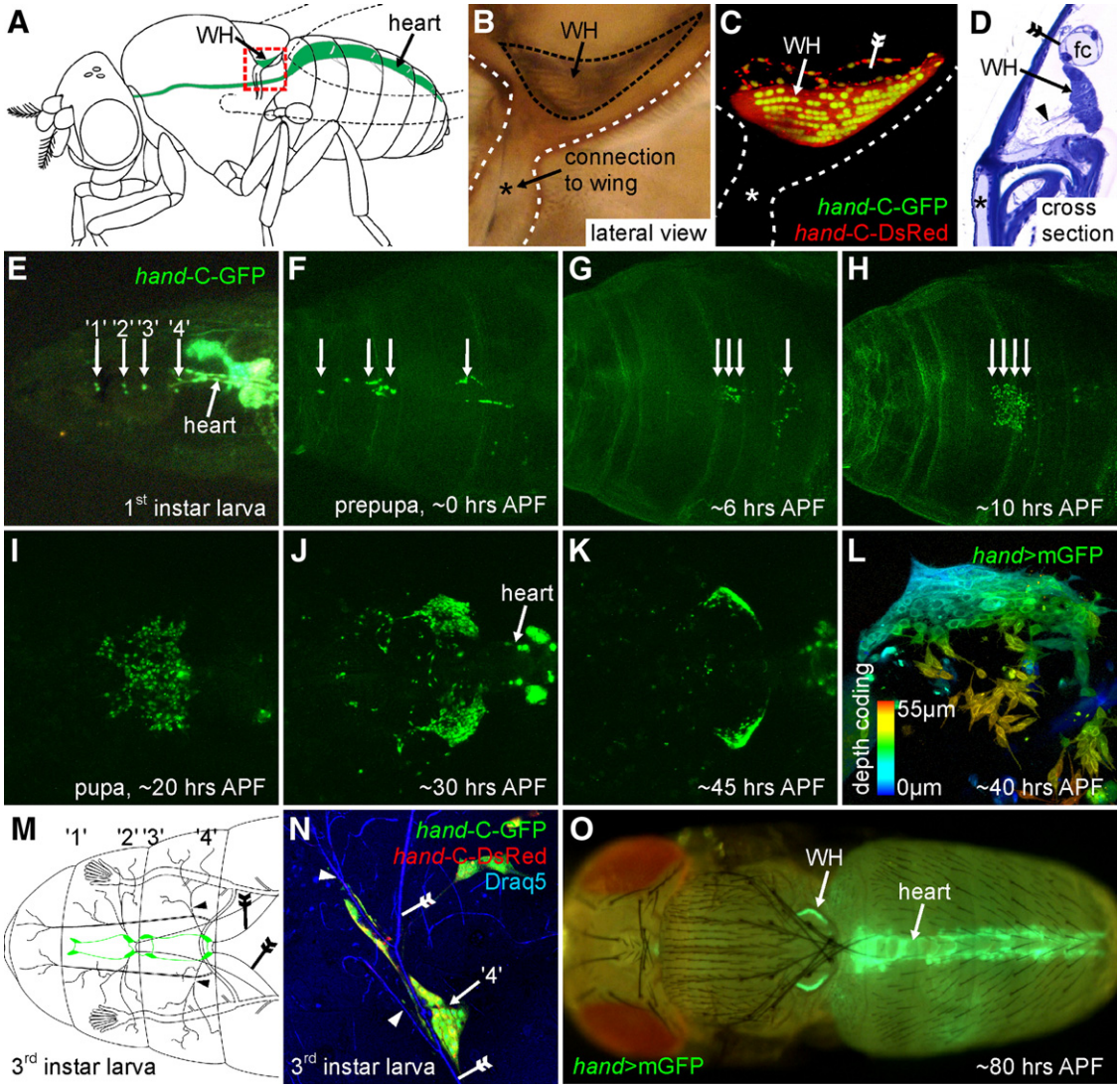

Fig. 1. Morphology and formation of wing hearts in postembryonic stages. (A) Diagram of an adult *Drosophila* showing the location of the left wing heart in relation to the heart. Encircled by red dots is the area depicted in B, C and D. (B) Bright field live image of left wing heart (encircled by black dots) and the tubular connection to the wing (outlined by the white dashed line in B and C, asterisk in B, C and D). (C) Confocal image of the left wing heart. The muscular portion forms horizontal rows, indicated by the nuclei, and is attached to a thin layer of cells which has a greater dorsal extension than the muscles (flagged line). Both tissues are labeled by the GFP reporter. (D) Muscular portion (arrow) and thin layer of cells (flagged line) in a histological cross section (1 µm thick, stained with toluidine blue; lateral is to the left). Fine strands suspend the wing heart to the lateral epidermis (arrowhead). A fat cell (fc) lies close to the wing heart. (E) Epifluorescence image of the anterior region of a first instar larva. From embryonic stage 16/17 on until the transition from the second to the third larval instar, the eight wing heart progenitors are arranged in four pairs (indicated by numbered arrows) in front of the heart. Proliferation in subsequent stages leads to clusters of cells still arranged in pairs. (F–H) Time-lapse study: Confocal images of the anterior region of a prepupa. Between about 1 h APF and about 10 h APF the four pairs of clusters merge into one single large cluster. (I–K) Time-lapse study: Confocal images of the thoracic region of a pupa (higher magnification as in F–H). At about 20 h APF the cluster splits longitudinally into two groups that migrate laterally and form the mature wing hearts over the next 30 h. (L) Confocal image of the forming right wing heart labeled by *hand*-C-Gal4>UASmCD8::GFP (membrane GFP) and depth coded at about 40 h APF. The fibroblast-like progenitors arrange themselves in an arched diaphragm. (M) Diagram of the anterior region of a third instar larva, illustrating the connection of the second and the fourth pair of clusters of progenitors to the dorsal tracheal branches in the second and third thoracic segment. Non relevant tracheae are not depicted for better clarity. Arrowheads indicate the part of the branch anterior to the interconnection between left and right branch and flagged lines the part of the branch posterior to the interconnection. (N) Confocal image of the fourth cluster of progenitors connected to the dorsal tracheal branches in a third instar larva (nuclei stained with Draq5). Tracheae are visible due to autofluorescence at 633 nm. Arrowheads and flagged lines like in M. (O) Epifluorescence image of a pharate adult, demonstrating the bilateral location of the wing hearts in the scutellum (labeled by *hand*-C-Gal4>UASmCD8::GFP).

transition from the second to the third larval instar, leading to eight clusters of cells that remain arranged in four pairs in the anterior region until 1 h after puparium formation (APF) (Fig. 1F). Between 1 and 10 h APF, the cell number increases significantly and the anterior three pairs of cell clusters are retracted to join the last pair of clusters, eventually forming one large median cluster (Figs. 1G, H; Supplementary data, Movie 3). Between 13 and 50 h APF, the single large cluster splits along its anterior–posterior axis into two groups of cells that migrate laterally in the forming scutellum, thereby adopting the characteristic arched appearance of the adult wing hearts (Figs. 1I–K; Supplementary data, Movie 4). During this process some of the cells on either side form the underlying thin layer while the remaining cells arrange in horizontal rows along that layer (Fig. 1L). First contractions of the mature organs were observed at about 45–50 h APF (this study and Sellin et al., 2006).

The wing heart progenitors are a subset of the Even-skipped pericardial cells

The expression of the bHLH transcription factor Hand in the wing heart progenitors, which serves as a general marker for all classes of heart cells in *Drosophila* (Han and Olson, 2005; Kölsch and Paululat, 2002), prompted us to screen for the expression of genes known to be active in cardiac lineages. Analysis of Even-skipped (Eve) expression

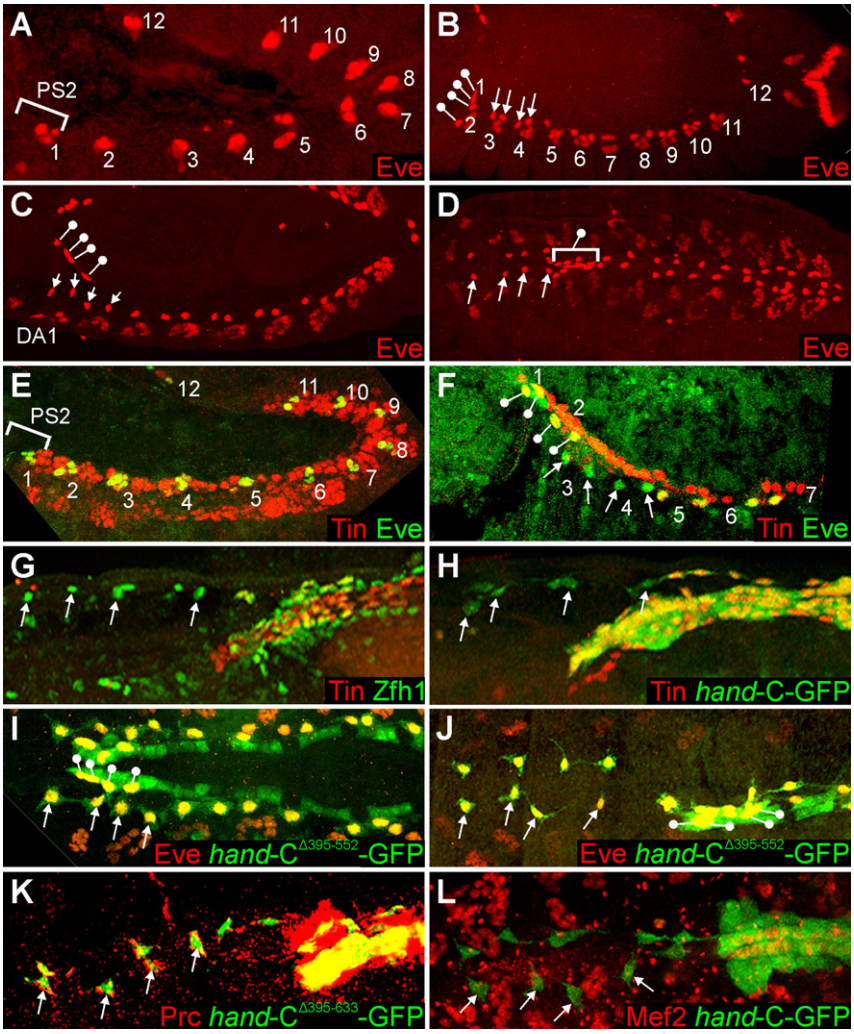

Fig. 2. Embryonic origin and specification of wing heart progenitors. EPCs originating from parasegments (PS) 2 and 3 are labeled by circled lines, those from PS 4 and 5 (wing heart progenitors) by arrows. (A) Lateral view of a stage 10 embryo. 12 Even-skipped expressing cell clusters appear in the cardiogenic mesoderm from PS 2 to 12 and 14. (B) Dorsal view of a stage 14 embryo. The pairs of EPCs that derive from PS 2 and 3 follow the cardioblasts (not stained) toward the midline of the embryo. The pairs of EPCs from PS 4 and 5 differentiate into the wing heart progenitors and are later located in front of the heart. Only the EPCs maintain Eve-expression whereas the sibling cell, the founder of the dorsal oblique muscle 2, loses Eve-expression. Instead, the founder of the dorsal acute muscle 1 (DA1) which does not share a common ancestor with the EPCs, expresses Even-skipped. (C) Dorsal view of a stage 14/15 embryo. The EPCs from PS 2 and 3 have almost reached the midline of the embryo, while the wing heart progenitors remain in close proximity to the developing DA1 muscle in their PS. (D) Dorsal view of a stage 16/17 embryo. The wing heart progenitors are now located in front of the heart, whereas the EPCs that originated in PS 2 and 3 are clustered at the anterior tip of the heart. (E) At stage 10 all 12 Eve clusters are Tinman positive. (F) Wing heart progenitor specification requires down-regulation of Tinman which occurs at about stage 13. At stage 14 the EPCs in PS 4 and 5 (arrows) are already Tinman negative while all the remaining EPCs maintain *tinman* expression. (G, H) Lateral view of stage 16/17 embryos. Down-regulation of Tinman persists in the wing heart progenitors, while it is expressed in all other EPCs. Pericardial cells are labeled by Zfh1 or *hand*-C-GFP. (I) Adjacent EPCs are interconnected by cytoplasmic extensions from parasegment 4 on, visualized by the *hand*-C$^{\Delta 395-552}$-GFP construct that mediates reporter gene activity in all Tinman positive cardioblasts and all EPCs. Although the GFP reporter gene has a nuclear localization signal, there is sufficient cytoplasmic GFP to visualize the shape of the cells. (J) During dorsal closure of the embryo, additional interconnections are established between opposing EPCs. (K) Lateral view of the anterior heart region of a stage 16/17 embryo. Pericardin, a marker for all known pericardial cells, is expressed in the wing heart progenitors shown by coexpression with the *hand*-C$^{\Delta 395-633}$-GFP construct that mediates reporter gene activity in all Tinman positive cardioblasts and all EPCs. (L) Dorsal view of the anterior heart region of a stage 16/17 embryo. Mef2, which stains cardiomyoblasts and somatic muscles cells, is absent from the wing heart progenitors, as well as from all other EPCs.

revealed that the embryonic wing heart progenitors arise through the same lineage as the well described Eve expressing pericardial cells (EPCs) (Carmena et al., 2002, 1998a,b; Han and Bodmer, 2003; Han et al., 2002; Park et al., 1998; Su et al., 1999). At stage 10 in embryogenesis, 12 Eve clusters are present on either side of the embryo, located in parasegments (PS) 2 to 12 (Han et al., 2002) and in PS 14 (this study). Each cluster gives rise to a pair of EPCs, except for the most posterior cluster in PS 14, which generates only one EPC (Figs. 2A, B). During subsequent development, the first and the second pair of EPCs, located in parasegment 2 and 3, turn toward the midline of the embryo to accompany the tip of the heart, which later bends ventrally into the embryo (Zikova et al., 2003) (Figs. 2B, C). The third and the fourth pair of EPCs in PS 4 and 5 are shifted anteriorly in relation to the heart. This step is not based on migration but on the remodeling of the embryo during head involution, since the cells remain in their PS close to the likewise Eve positive anlagen of the DA1 muscle (Fig. 2C). The EPCs in PS 4 and 5 subsequently differentiate into the later wing heart progenitors, while all others become the classical EPCs and accompany the heart in a loosely associated fashion. At least from PS 4 to 12, all pairs of Eve positive cells (wing heart progenitors and classical EPCs) are interconnected by cytoplasmic extensions (Figs. 2I, J) forming a rope ladder-like strand above the heart after dorsal closure at stage 16/17. This mode of contact between the cells persists in the wing heart progenitors in postembryonic stages (data not shown) and might be essential for proper relocation in the prepupae.

To verify the role of *eve* in wing heart formation, we used a rescue transgene which removes *eve* activity in the mesoderm, while completely rescuing the phenotype of *eve* null mutants in all

other embryonic tissues (Fujioka et al., 2005). In embryos of this genotype all EPCs and wing heart progenitors fail to differentiate (Figs. 3A, B). Although these animals were reported to survive into adults (Fujioka et al., 2005), we were only able to observe larval stages under our breeding conditions, all of which lacking the wing heart progenitors as revealed by the *hand*-C-GFP reporter (data not shown). In a previous study, *zfh1* was shown to be required for EPC differentiation, suggesting that *zfh1* acts as a direct activator of *eve* in EPCs (Su et al., 1999). We found that the described lack of *eve* expression in the majority of EPCs (Su et al., 1999) in embryos homozygous mutant for *zfh1*, includes the wing heart progenitors (Figs. 3C, D), underlining that wing heart progenitors share a common lineage with EPCs. Consistently, the correct specification of the embryonic wing heart progenitors depends on factors involved in lateral inhibition or asymmetric cell division: Like the classical EPCs, they are reduced in number in *kuzbanian* or *mastermind* mutants (Albrecht et al., 2006 and data not shown), reduced or almost absent in *inscuteable* mutants (Fig. 3J), and about doubled in number in *numb* mutant embryos (Fig. 3K). In homozygous mutant *pavarotti* embryos, in which cytokinesis is blocked in heart cells and other tissues, the siblings that arise from a common progenitor remain attached to each other. In such mutants, the classical EPCs and the wing heart progenitors are both affected, further corroborating that wing heart progenitors are a specialized subset of EPCs (Figs. 3G, H).

Genetic control of wing heart progenitor specification

So far, *hand* is the only gene we could identify that is continuously expressed in the wing hearts from the embryonic progenitors onward in all developmental stages and in the adult fly. Embryos which are homozygous mutant for *hand* display a normal number of wing heart progenitors located at the correct position anterior to the heart (Figs. 3E, F). We therefore conclude that, as for the initial visceral mesoderm differentiation (Popichenko et al., 2007; Varshney and Palmer, 2006) and the determination and specification of heart cells (Lo et al., 2007),

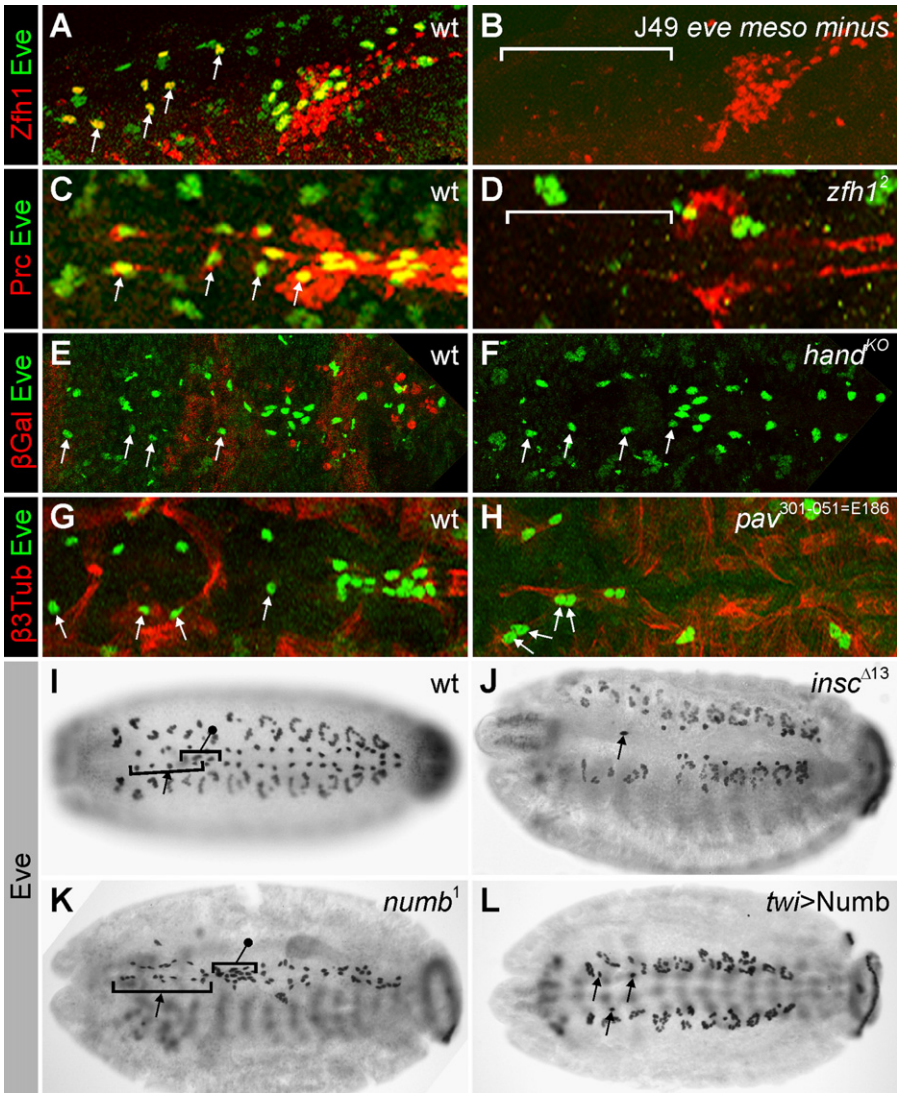

Fig. 3. Wing heart development in selected mutant background. Differentiation of the wing heart progenitors (arrows) depends on Eve and Zfh1 but not on Hand. In stage 16/17 embryos mutant for the genes *eve* (A, B) and *zfh1* (C, D), the majority of EPCs, including the wing heart progenitors (brackets), fail to differentiate. In contrast, *hand* which is expressed continuously in the wing hearts from embryonic stages is dispensable. In homozygous knock out mutants (indicated by the lack of the β-Gal balancer staining), all wing heart progenitors and EPCs are present (E, F). In *pavarotti* mutants (G, H), EPCs including the wing heart progenitors are present but the siblings remain attached to each other due to blocked cytokinesis at cycle 14/15. (I–L) EPCs originating from parasegments (PS) 2 and 3 are labeled by circled lines, those from PS 4 and 5 (wing heart progenitors) by arrows. The number of wing heart progenitors and the number of classical EPCs is reduced in *inscuteable* mutants (J) and increased in *numb* (K) mutant embryos, indicating that both arise throughout asymmetric cell division. Overexpression of Numb in the whole mesoderm using *twi*-Gal4 results in the loss of almost all wing heart progenitors and classical EPCs (L). This mimics the effect of the *inscuteable* mutant which is a switch from asymmetric to symmetric cell division resulting in two founders for the dorsal oblique muscle 2 and no EPC progenitor per hemisegment.

hand is not essential for embryonic specification and differentiation of the wing heart progenitors.

To identify factors that promote the identity of the embryonic wing heart progenitors within the lineage of EPCs, we investigated the expression of known pericardial cell markers such as Zfh1, Pericardin and Tinman. We found that, like in all classical EPCs (Chartier et al., 2002; Han et al., 2002), *zfh1* and *pericardin* are expressed in all wing heart progenitors at stage 16/17 (Figs. 2G, K and 3A, C). Furthermore, our data suggest that the previously reported unidentified Pericardin positive cells located anterior to the heart (Chartier et al., 2002) correspond to the wing heart progenitors. In contrast, *tinman* expression is excluded from the eight progenitor cells from about stage 13 on, prior to their relocation to the front of the heart (Figs. 2E–H), while it persists in the classical EPCs until stage 16/17 (this study and Han et al., 2002). Since mature wing hearts consist of contractile muscle cells, we also tested the progenitors for expression of Mef2 (Fig. 2L), β3Tubulin (Fig. 3G), and Seven-up (data not shown), factors that are expressed in the contractile cardiomyoblast lineage. None of them were found to be active in the embryonic wing heart progenitors at stage 16/17. Thus, so far, down-regulation of Tinman is the only identified signal involved in the specification of the embryonic wing heart progenitors.

Laser ablation and ectopic expression of Tinman in wing heart progenitors

To prove that the identified eight embryonic cells are indeed the anlagen of the wing hearts, we used laser ablation to eliminate them in stage 16/17 embryos. After ablation, no GFP signal from progenitors could be detected in larval or pupal stages, indicating omitted wing heart formation (Figs. 4E, F). The lack of wing hearts was confirmed by histological sections (data not shown). To investigate whether the absence of *tinman* expression in the wing heart progenitors at late stages is indeed of importance for their cell fate, we used the UAS-GAL4 system to ectopically drive Tinman expression in these cells in conjunction with a GFP reporter. Individuals with the genotype *hand*-C-Gal4>UAS-Tin,UAS-mGFP or *eme*-Gal4>UAS-Tin,UAS-eGFP (Figs. 4A–D) were examined at late pupal stages for the presence or absence of the wing hearts. We found that 100% ($n=31$) of the flies lacked wing hearts when the strong *hand*-C-Gal4 driver was used, while 46% ($n=63$) of the individuals had no wing hearts in the case of the weaker *eme*-Gal4 driver (Supplementary data, Table 2). Absence of wing hearts was also confirmed by histological sections (data not shown). We conclude that loss of Tinman activity is crucial for acquiring wing heart progenitor cell fate.

Wing hearts are essential for wing maturation

Adults lacking wing hearts, obtained either from laser ablation of wing heart progenitors or ectopic expression of Tinman in the wing heart progenitors, exhibit a severe wing phenotype. Unfolding of the wings takes place normally in these individuals, indicating that hemolymph influx into the wings is not affected. However, the wings of individuals lacking wing hearts retain the typical opaque appearance inherent to newly eclosed flies and do not become clear within the first 3 h as in wild-type flies (Fig. 4). A flight assay revealed that none of these individuals were able to fly (Table 1). To exclude side effects of the UAS-Gal4 system or of the laser irradiation in the embryo, we ablated the wing hearts in pharate adults on only one side (Figs. 5A, B) to generate individuals with an internal control. Adults obtained from this experiment displayed one opaque and one clear wing 3 h after wing unfolding corresponding to the side of ablation

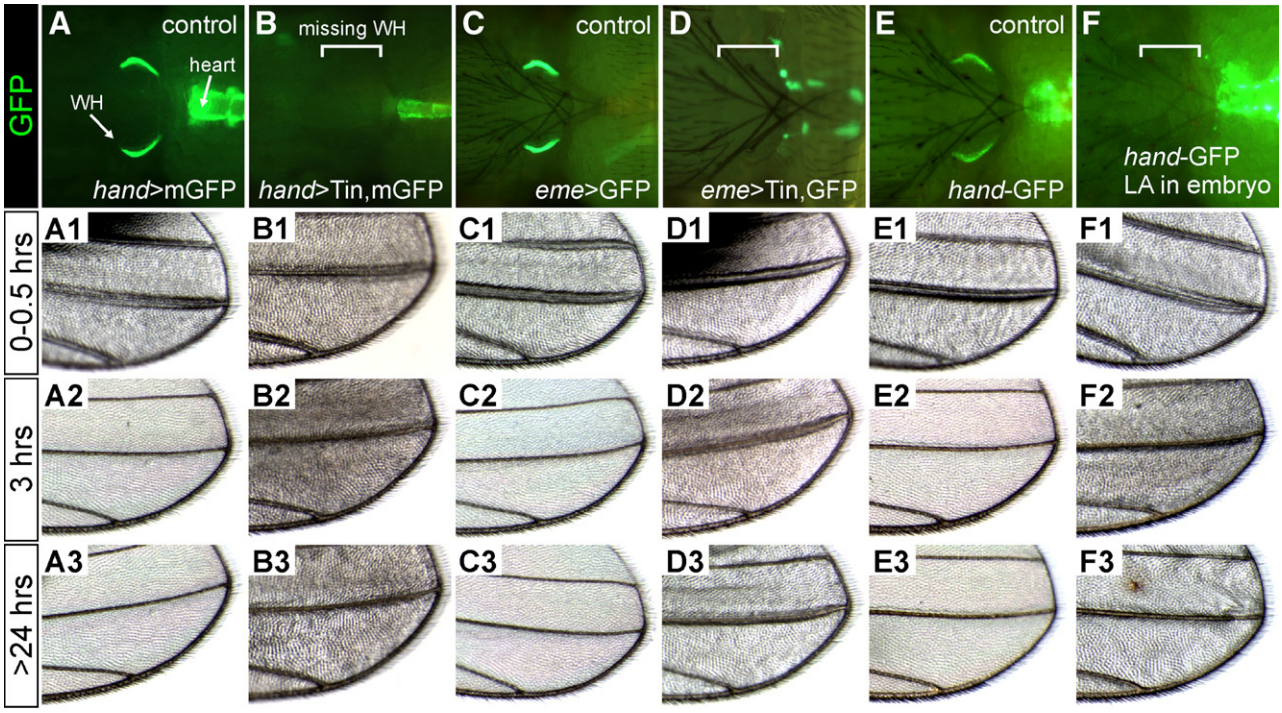

Fig. 4. Loss of wing hearts results in a severe wing phenotype. Viable adults lacking wing hearts (WH) were obtained from three independent experimental setups: (A, B) Ectopic expression of Tinman using the *hand*-C-Gal4 driver (pupal expression in wing hearts and heart). (C, D) Ectopic expression of Tinman using the *eme*-Gal4 driver (pupal expression in wing hearts and some somatic muscles). (E, F) Laser ablation (LA) of the wing heart progenitors in stage 16/17 embryos (*hand*-C-GFP labeled, pupal expression in wing hearts and heart). All three experiments show identical results: Shortly (0–0.5 h) after wing unfolding no difference is visible between adults lacking wing hearts and the corresponding controls. The wings are fully extended and exhibit a slightly opaque appearance. About 3 h after wing unfolding the wings become clear in the controls but remain slightly opaque in animals lacking wing hearts. This condition is retained throughout the complete life span. The difference in the opacity of the wings between individuals without and individuals with wing hearts is caused by the different scattering of light when passing through bonded (controls) or unbonded wing surfaces. In the latter, the wings are still filled with hemolymph and the cuticle of each epidermal cell forms a small elevation with a bristle at its top. In contrast, the cuticles of bonded wings are flat and only the pattern of the bristles is visible. Epifluorescence images and bright field images originate from different individuals.

Table 1
Effect of loss of wing hearts on wing maturation and flight ability

Genotype			Opaque wings 3 h AWU	Unable to fly
hand-C-Gal4>UASTin,UAS-mGFP	at 22 °C	(*n*=54)	54=100%	54=100%
eme-Gal4>UAS-Tin,UASeGFP	at 29 °C	(*n*=29)	29=100%	29=100%
LA in embryo: *hand*-C-GFP;*hand*-C-DsRed	at 22 °C	(*n*=16)	16=100%	16=100%
LA in pupa: *hand*-C-GFP;*hand*-C-DsRed	at 22 °C	(*n*=5)	5=100%	5=100%
Control: *hand*-C-Gal4>UAS-mGFP	at 22 °C	(*n*=51)	0=0%	1=2%
Control: *eme*-Gal4>UAS-eGFP	at 29 °C	(*n*=51)	0=0%	1=2%
Control: *hand*-C-GFP;*hand*-C-DsRed	at 22 °C	(*n*=36)	2=6%	2=6%

Ectopic expression of *tinman* in wing heart progenitors, laser ablation (LA) of progenitors in the embryo, or one-sided LA of mature wing hearts in late pupae results in viable individuals lacking wing hearts. The weaker *eme*-Gal4 driver was used at 29 °C to enhance its performance. All adults obtained from these experiments still exhibited opaque wings 3 h after wing unfolding (AWU) and were unable to fly. Individuals from one-sided ablation show only one opaque wing corresponding to the side of ablation. On the untreated side, the wing becomes clear 3 h AWU (not shown).

(Table 1). Histological sections through the wings showed that, while on the intact side the dorsal and the ventral cuticles were tightly bonded forming a flexible wing blade, they remained apart on the side of ablation and the wing was still filled with hemolymph (Figs. 5C–F). To test whether wing hearts are involved in wing maturation, we generated flies with labeled wing hearts (*hand*-C-DsRed) and labeled wing epidermal cells [*30A*-Gal4>UAS-eGFP (Kiger et al., 2007)]. One-sided ablation of wing hearts in pupae of these flies revealed that delaminated epidermal cells are only cleared from the wing during maturation on the intact side, corresponding to the activity of the wing heart (Figs. 5G–I; Supplementary data, Movies 5 and 6). On the side of ablation, GFP fluorescence was still visible in the wing after 24 h, although the epidermal cells had delaminated as indicated by their disarrayed pattern.

Discussion

Although the *Drosophila* wing hearts are known for many years (Miller, 1950), their origin and development remained unknown so far. Here, we provide the first developmental approach on these organs using *in vivo* time lapse imaging as well as genetic and immunohistochemical methods. We found that the wing hearts develop from embryonic anlagen that consist of eight progenitor cells located anterior to the heart. Analysis of gene expression in these progenitors confirmed the hypothesis that the wing hearts originate from the cardiac mesoderm (Lawrence, 1982), but not from the contractile cardioblast lineage, as was suggested based on anatomical data (Krenn and Pass, 1995). Surprisingly, the embryonic anlagen derive from a particular subset of the well-known EPCs. EPCs arise in pairs in PS 2 to 12 from the dorsal progenitor P2, which divides asymmetrically into the founder of the dorsal oblique muscle 2 and the founder of the EPCs in a *numb*-dependent lineage decision (Carmena et al., 2002, 1998a,b; Han and Bodmer, 2003; Park et al., 1998). Additionally, a single EPC arises in PS 14 (this study). The subsequent differentiation of the founders into EPCs requires the activity of the transcription factors Zfh1 and Eve (Fujioka et al., 2005; Han et al., 2002; Su et al., 1999). In this study, we show that the EPCs located in PS 4 and 5 are relocated in relation to the heart during head involution at stage 14/15 of embryogenesis and subsequently differentiate into the wing heart progenitors. Until this step, no difference to the EPCs in the anterior and posterior PS could be detected. Like the classical EPCs, which remain close to the heart, the EPCs that give rise to the wing heart progenitors depend on factors involved in asymmetric cell division, e.g. Insc or Numb, and fail to differentiate in embryos mutant for *zfh1* as well as in animals lacking mesodermal Eve. Loss of *tinman* expression is the only event we could identify that discriminates between a classical EPC fate and the specification of wing heart progenitors. Consistently, ectopic expression of Tinman in the wing heart progenitors effectively represses their specification, probably by committing them to a classical EPC fate, indicating that Tinman plays a crucial role in the involved regulatory pathway.

So far, the biological role of pericardial cells (PCs), and EPCs in particular, is not well understood. In the embryo, three populations of PCs arise in each segment, which are characterized by the expression of different combinations of genes (Odd positive PCs, Eve positive PCs, and Tinman positive PCs) (Ward and Skeath, 2000). During postembryonic stages, the number of PCs decreases (Das et al., 2008b; Sellin et al., 2006), raising the question which population contributes to the

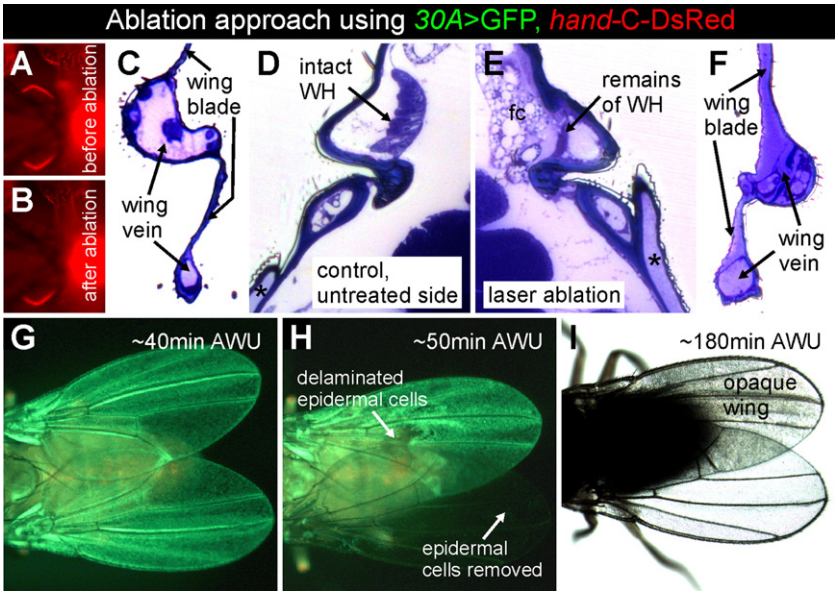

Fig. 5. The role of wing hearts in wing maturation. (A, B) One-sided laser ablation of a *hand*-C-DsRed labeled mature wing heart (WH) in a late pupa. After ablation, no signal from the wing heart is detectable indicating successful destruction of the tissue. (C–F) Histological cross sections (1 μm thick, stained with toluidine blue) through an adult fly after one-sided ablation of a wing heart (asterisk indicates the tubular connection to the wing). On the untreated side, the wing surfaces are tightly bonded (C) and the wing heart is intact (D), while on the side of ablation, only remains of the wing heart are present (E) and the wing surfaces remain apart (F). (G–I) Removal of epidermal cells (labeled by *30A*-Gal4>UAS-eGFP) from the wings starts about 40 min after wing unfolding (AWU) only on the untreated side (bottom) resulting in a clear wing 180 min AWU. Although the epidermal cells delaminate on the side of ablation (top), as indicated by their disarrayed pattern, they remain in the wing which retains its opaque appearance.

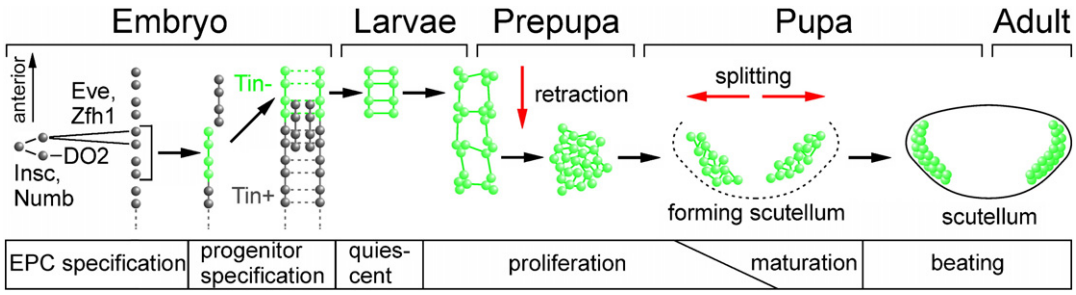

Fig. 6. Model of wing heart development in *Drosophila*. A pair of EPCs and the dorsal oblique muscle 2 (DO2) arise from a common progenitor [shown for parasegment (PS) 4] in each of the PS 2 to 12 on either side of the embryo (only PS 2 to 6 of one side are shown). EPC specification depends on Insc and Numb as well as on the transcription factors Zfh1 and Eve. Subsequently, adjacent EPCs are interconnected by cytoplasmic extensions. The wing heart progenitors derive from the EPCs in PS 4 and 5 (bracket) and are specified by loss of Tinman expression at about stage 13. The anterior two pairs of EPCs remain at the tip of the heart (not shown) while the wing heart progenitors are relocated anterior to the heart during head involution (both sides are shown). At the end of embryogenesis, the eight progenitors are arranged in four pairs and additional interconnections between opposing cells have been established. Until the transition from the second to the third larval instar the progenitors remain quiescent. In the subsequent proliferation phase, all progenitors divide to form eight clusters of cells. During the prepupal stage, the clusters are relocated and merge into one large cluster. In the subsequent pupal stage, this cluster splits longitudinally and the cells migrate laterally into the forming scutellum. During the final maturation phase, the muscle cells are attached to the thin layer of cells and the wing hearts adopt their typical arched appearance. Beating starts at late pupal stage and persists into the adult.

final set of PCs in the adult and whether all PCs have the same function throughout development. Recent studies have shown that postembryonic PCs express Odd and Eve, a combination which is not observed in the embryo (Das et al., 2008b), and are dispensable for cardiac function. Genetic ablation of all larval PCs had no effect on heart rate, but increased sensitivity to toxic stress (Das et al., 2008a). In contrast, the specification of the correct number of embryonic PCs is crucial for normal heart function. Loss of mesodermal Eve during embryogenesis results in fewer larval pericardial cells, which causes a reduction in heart rate and lifespan (Fujioka et al., 2005). Conversely, hyperplasia of embryonic PCs has no effect on heart rate but causes decreased cardiac output. This was explained by an excess of Pericardin secreted by the PCs into the extracellular matrix enveloping the heart (Johnson et al., 2007). Taken together, embryonic PCs seem to influence cardiac development by e.g., secreting substances whereas postembryonic PCs function as nephrocytes. However, in this study, we provide the first functional data on a subset of embryonic EPCs, which differentiate into adult progenitors giving rise to a myogenic lineage. This represents a completely new function of PCs, raising the question whether EPCs might in general have myogenic potential and rather represent a population of adult progenitors, than PCs in a functional sense.

The organogenesis of the wing hearts is a highly dynamic process (summarized in Fig. 6), which includes distinct cellular interactions. At first, adjacent EPCs (including the wing heart progenitors) on either side of the embryo establish contact via cytoplasmic extensions. After dorsal closure of the embryo, interconnections are also formed between opposing EPCs resulting in a rope ladder-like strand above the heart. We assume that these interconnections are needed to retain contact between the wing heart progenitors during the subsequent development. During larval stages, some of the wing heart progenitors establish a second contact to specific tracheal branches and proliferation starts. In the prepupa, a relocation event joins all wing heart progenitors in one large cluster. During this step, the progenitors are probably passively relocated in conjunction with the tracheal branches to which they are connected. Finally, the wing heart progenitors initiate active migration and form the mature wing hearts in the pupa. Considering the complexity of their development, we propose that wing hearts provide an ideal model for studying organogenesis on several different levels such as signaling, cell polarity, or path finding.

Elimination of the embryonic progenitors by ectopic expression of *tinman* or by laser ablation causes the loss of wing hearts, which results in a specific wing phenotype in conjunction with flightlessness. In the identified phenotype, the delaminated epidermal cells are not cleared from the wings during wing maturation and bonding of the dorsal and ventral wing surfaces is omitted. Recently, it was reported that the epidermal cells transform into mobile fibroblasts and actively migrate out of the wings (Kiger et al., 2007). However, in our *in vivo* time-lapse studies we could not observe migration of epidermal cells during wing clearance. Conversely, their movements correlated with the periods of wing heart beating, indicating that they are passively transported by the hemolymph flow. One-sided ablation of mature wing hearts in pupae, confirms that wing hearts play a crucial physiological role in wing maturation, since the wing phenotype occurs only on the treated side, but in the same genetic background. In contrast, mutations in genes coding for proteins involved in cell adhesion, e.g. integrins, or in adhesion to the extra cellular matrix, cause a blistered wing phenotype (Bökel et al., 2005; Brabant et al., 1996; Brower and Jaffe, 1989; Prout et al., 1997; Walsh and Brown, 1998). In the latter phenotype, the epidermal cells of the immature wings are not attached to their opposing cells or to the cuticle and the wing surfaces are separated during unfolding by the sudden influx of hemolymph. In contrast, in animals lacking wing hearts the wings resemble those of the wild-type shortly after unfolding. The epidermal cells also delaminate later from the cuticle, as indicated by their disarrayed pattern, but are not removed from the wings due to the missing hemolymph circulation and probably impede spatially the bonding of the dorsal and the ventral cuticle. Thus, the wings remain in their immature state and do not acquire aerodynamic properties, which accounts for the flightlessness. We conclude that wing hearts are crucial for establishing proper wing morphology and functionality in *Drosophila*.

Wing hearts occur in all winged insects, but differ considerably in their morphology. However, their function is highly conserved, since they all function as suction pumps that draw hemolymph from the wings (Pass, 2000). In the basal condition, the heart itself is directly connected to the scutellum and constitutes the pump (Krenn and Pass, 1994). This connection was lost several times during evolution (Krenn and Pass, 1995) and other muscles, e.g. the separate wing hearts in *Drosophila*, were recruited to retain the function indicating a high selection pressure on wing circulation. We suggest that this is due to the crucial role of wing hearts during wing maturation. Since proper wing morphogenesis is essential for flight ability, insect flight might not have been possible before the evolution of wing hearts.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.02.043.

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